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CHIMERIC GABA_B RECEPTOR

The present invention provides a novel method to identify substances that are agonists of GABA_B receptors, using a ³H-GABA binding assay in recombinant
5 GABA_BR1a/R2 receptor expressing cells.

BACKGROUND OF THE INVENTION

GABA (γ-amino-butyric acid) is the most widely distributed amino acid inhibitory neurotransmitter in the central nervous system (CNS) activating two distinct
10 families of receptors; the ionotropic GABA_A and GABA_C receptors for fast synaptic transmissions, and the metabotropic GABA_B receptors governing a slower synaptic transmission.

GABA_B receptors are members of the superfamily of seven transmembrane G-protein coupled receptors that are coupled to neuronal K⁺ or Ca²⁺ channels.
15 Presynaptic GABA_B receptor activation has generally been reported to result in the inhibition of Ca²⁺ conductance, leading to a decrease in the evoked release of neurotransmitters. Post-synaptically the major effect of GABA_B receptor activation is to open potassium channels, to generate post-synaptic inhibitory potentials.

The expression of GABA_B receptors is widely distributed in the mammalian neuronal axis, with particularly high levels in the molecular layer of the cerebellum,
20 interpeduncular nucleus, frontal cortex, olfactory nuclei, thalamic nuclei, temporal cortex, raphe magnus and spinal cord. GABA_B receptors are also present in the peripheral nervous system, both on sensory nerves and on parasympathetic nerves. Their ability to modulate these nerves give them potential as targets in disorders of the
25 lung, GI tract and bladder (Belley et al., 1999, Biorg. Med. Chem. 7:2697-2704).

A large number of pharmacological activities have been attributed to GABA_B receptor activation, such as for example, analgesia, hypothermia, catatonia, hypotension, reduction of memory consolidation and retention, and stimulation of insulin, growth hormone and glucagon release (see Bowery, 1989, Trends Pharmacol.
30 Sci. 10:401-407 for a review). It is well accepted that GABA_B receptor agonists and antagonists are pharmacologically useful in indications such as stiff man syndrome, gastroesophageal reflux, neuropathic pain, incontinence and treatment of cough and cocaine addiction. For example, the GABA_B receptor agonist baclofen has been shown to reduce transient lower esophageal sphincter relaxations (TLESR) and is accordingly
35 useful in the treatment of reflux as most episodes of reflux occur during TLESR. However, the current GABA_B receptor agonists, such as baclofen, are relatively non-

selective and show a variety of undesirable behavioural actions such as sedation and respiratory depression. It would be desirable to develop more GABA_B receptor agonists with an improved selectivity and less of the aforementioned undesirable effects.

5 Current methods of drug discovery generally involve assessing the biological activity of tens or hundreds of thousands of compounds in order to identify a small number of those compounds having a desired activity against a particular target, i.e. High Throughput Screening (HTS). In a typical HTS related screen format, assays are performed in multi-well microplates, such as 96, 384 or 1536 well plates, putting
10 certain constraints to the setup of the assay to be performed including the availability of the source materials (i.e membrane preparations of cells expressing the recombinant GABA_B receptor). HTS related screens are preferably performed at room temperature with a single measurement for each of the compounds tested in the assay, requiring short cycle times, with a reproducible and reliable output.

15 Present *in vitro* screens to identify compounds as agonists of the GABA_B receptor, either rely on natural, less abundant resources such as binding assays in rat brain membranes or consist of functional screening assays, such as for example Ca²⁺ responses, c-AMP responses and effects on Ca²⁺ and K⁺ channels performed in cells expressing a recombinant GABA_B receptor. In some of these functional assays the
20 GABA_B receptors may be co-expressed with G-proteins, e.g. Gα16 or Gqi5 or the chimeric G-protein G αq-z5, increasing G-protein coupling (Bräuner-Osborne & Krogsgaard-Larsen, 1999, Br. J. Pharmacol. 128:1370-1374). However, a GABA_B agonist binding assay that would further reduce the HTS cycle time and the resources for biochemicals such as recombinant proteins, is currently unavailable.

25 The present invention describes the development of a Chinese Hamster Ovary (CHO) cell line co-expressing the human GABA_B receptor subunits GABA_BR1a and GABA_BR2, which were surprisingly found to demonstrate agonist binding in radioligand binding experiments. In addition, the present inventors demonstrated that the hGABA_BR1a/GABA_BR2 CHO cell line has one high affinity and one low affinity
30 agonist binding site in the recombinant expressed GABA_B receptor. Hence the hGABA_BR1a/GABA_BR2 CHO cell line provided by the present invention not only allows compound screening, but also provides a useful tool to characterize the nature of the compound –receptor interaction.

SUMMARY OF THE INVENTION

The present invention provides an isolated GABA_B receptor protein comprising at least one GABA_BR1a subunit and at least one GABA_BR2 subunit, characterized in that said GABA_B receptor has one high affinity agonist binding site and one low affinity agonist binding site. In particular the isolated recombinant GABA_B receptor protein expressed by the hGABA_BR1a/GABA_BR2 CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone on August 22, 2003 with the accession number LMBP 6046CB. It is thus an object of the present invention to provide the hGABA_BR1a/GABA_BR2 CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone on August 22, 2003 with the accession number LMBP 6046CB.

The invention also provides the use of the aforementioned cell line in a method to identify GABA_B receptor agonists using a functional or a binding assay. In particular in a radioligand-binding assay comprising the use of radiolabeled agonists such as for example ³H-GABA or ³H-baclofen.

The invention further provides a method to identify GABA_B receptor agonists, comprising contacting the aforementioned cell line with a test compound and measuring the binding of said test compound to the GABA_B receptor. In particular the method consists of a radioligand binding assay, comprising exposing the aforementioned cells to a labelled agonist of GABA_B in the presence and absence of the test compound and measure the binding of the labelled ligand to the cells according to the invention, where if the amount of binding of the labelled ligand is less in the presence of the test compound, then the compound is a potential agonist of the GABA_B receptor.

It is also an object of the present invention to provide a method to identify a high affinity GABA_B receptor agonist, said method comprising contacting the aforementioned cells with the radiolabeled agonist selected from the group consisting of GABA, baclofen and 3-aminopropylphosphinic acid (3-APPA a.k.a APMPA), in the presence and absence of the test compound and measure the binding of the labelled ligand to the cells according to the invention, where if the amount of binding of the labelled ligand to the high affinity binding site is less in the presence of the test compound, then the compound is a potential high affinity agonist of the GABA_B receptor.

Alternatively, the aforementioned binding assays are performed on cellular extracts, in particular cellular membrane preparations of the hGABA_BR1a/GABA_BR2 CHO cell line deposited at the Belgian Coordinated

Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone on August 22, 2003 with the accession number LMBP 6046CB.

5 In another embodiment the present invention provides a method to identify a GABA_B receptor agonist, said method comprising contacting the aforementioned cell line with a compound to be tested and determine whether the compound activates a GABA_B receptor functional response in said cells. In particular the functional response consists of modulation of the activity of ion channels or of intracellular messengers as explained hereinafter.

10 This and further aspects of the present invention will be discussed in more detail hereinafter.

BRIEF DESCRIPTION OF THE DRAWING

15 Figure 1 GTP γ 35S-binding upon stimulation of membranes by GABA expressed as the percentage of maximal GABA stimulation, in the presence and absence of the positive allosteric modulator CGP7930>

20 Figure 2 Displacement of ³H-GABA by agonists (baclofen, GABA & APMPA) and antagonists (SCH50911 & CGP54626)

Figure 3 Reproducible agonist IC₅₀ values (n=5) independent of membrane preparations.

25 Figure 4 Two sided ³H-GABA agonist binding curve in the presence or absence of 10 μ M CGP54626 (a) or JNJ 4309747-AAD (b).

DETAILED DESCRIPTION

30 For the purposes of describing the present invention: GABA_BR1a or h GABA_BR1a as used herein refers to the human GABA_B receptor subunit known as GABA_BR1a in Kaupmann et al, 1998, Proc. Natl. Acad. Sci. USA 95:14991-14996, the amino acid sequence (SEQ ID No.:2) of which can be found at GenBank Accession no. AJ225028, as well as to its mammalian orthologs. GABA_BR1a also refers to other GABA_B receptor subunits that have minor changes in amino acid sequence from those described

hereinbefore, provided those other GABA_B receptor subunits have substantially the same biological activity as the subunits described hereinbefore. A GABA_BR1a subunit has substantially the same biological activity if it has an amino acid sequence that is at least 80% identical to, preferably at least 95% identical to, more preferably at least 97% identical to, and most preferably at least 99% identical to SEQ ID No.: 2 and has a K_d or EC₅₀ for GABA, GABA_B receptor agonists such as for example baclofen and gabapentin or GABA_B receptor antagonists such as for example CGP54626A, SCH 50911, saclofen and phaclofen, that is no more than 5-fold greater than the K_d or EC₅₀ of a native GABA_B receptor for GABA or the same GABA_B receptor agonist or GABA_B receptor antagonist.

GABA_BR2 as used herein refers to the human GABA_B receptor subunit known as GABA_BR2 in White et al., 1998, Nature 396:679-682, the amino acid sequence (Seq Id NO.: 4) of which can be found at GenBank accession no. AF058795 as well as to its mammalian orthologs. GABA_BR2 also refers to other GABA_B receptor subunits that have minor changes in amino acid sequence from those described hereinbefore, provided those other GABA_B receptor subunits have substantially the same biological activity as the subunits described hereinbefore. A GABA_BR2 subunit has substantially the same biological activity if it has an amino acid sequence that is at least 80% identical to, preferably at least 95% identical to, more preferably at least 97% identical to, and most preferably at least 99% identical to SEQ ID No.: 4 and has in combination with a GABA_BR1 subunit a K_d or EC₅₀ for GABA, GABA_B receptor agonists such as for example baclofen and gabapentin or GABA_B receptor antagonists such as for example CGP54626A, SCH 50911, saclofen and phaclofen, that is no more than 5-fold greater than the K_d or EC₅₀ of a native GABA_B receptor for GABA or the same GABA_B receptor agonist or GABA_B receptor antagonist.

The K_d and EC₅₀ values of the native GABA_B receptor is determined using the methods known to a person skilled in the art, in particular using competition binding studies on tissue preparations such as for example described in Cross & Horton, 1987 Eur.J.Pharmacol. 141(1): 159-162. Briefly, crude synaptic membranes are prepared by homogenisation of whole brain, centrifugation (30 000 xg, 20 min.) and extensive washing. Total binding is measured by incubation of the membranes with ³H-GABA or ³H-baclofen, while non-specific binding is measured in the presence of 100 μM baclofen. Upon removal of unbound ligand by filtration, filters are counted in a β-counter or a Topcount Harvester (Packhard). For competition experiments the binding occurs in the presence of increasing concentration of unlabeled compound.

It is thus an object of the present invention to provide an isolated GABA_B receptor protein formed by at least one GABA_BR1a and at least one GABA_BR2 subunit further characterized in that said isolated GABA_B has both a high and a low affinity agonist binding site. In a further embodiment this isolated GABA_B receptor is a functional GABA_B receptor expressed by a cell, wherein said cell does not normally express the GABA_B receptor. Suitable cells which are commercially available, include but are not limited to L-cells, HEK-293 cells, COS cells, CHO cells, HeLa cells and MRC cells, in particular CHO cells wherein the GABA_B receptor protein comprises at least one GABA_BR1a subunit encoded by the oligonucleotide sequence consisting of SEQ ID No.1 and at least one GABA_BR2 subunit encoded by the oligonucleotide sequence consisting of SEQ ID No.3. In a more particular embodiment the isolated GABA_B receptor according to the invention, consists of the receptor protein expressed by the hGABA_BR1a/GABA_BR2 CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with accession number LMBP 6046CB.

"Functional GABA_B receptor" refers to a GABA_B receptor formed by co-expression of GABA_BR2 and GABA_BR1a in a cell, wherein said cell does not normally express the GABA_B receptor, most preferably resulting in a heterodimer of GABA_BR2 and GABA_BR1a, where the functional GABA_B receptor mediates at least one functional response when exposed to the GABA_B receptor agonist GABA. Examples of functional responses are : pigment aggregation in *Xenopus melanophores*, negative modulation of cAMP levels, coupling to inwardly rectifying potassium channels, mediation of late inhibitory postsynaptic potentials in neurons, increases in potassium conductance, decreases in calcium conductance, MAPKinase activation, extracellular pH acidification, and other functional responses typical of G-protein coupled receptors. One skilled in the art would be familiar with a variety of methods of measuring the functional responses of G-protein coupled receptors such as the GABA_B receptor (see, e. g., Lerner, 1994, *Trends Neurosci.* 17: 142-146 [changes in pigment distribution in melanophore cells]; Yokomizo et al., 1997, *Nature* 387: 620-624 [changes in cAMP or calcium concentration; chemotaxis]; Howard et al., 1996, *Science* 273: 974-977 [changes in membrane currents in *Xenopus oocytes*]; McKee et al., 1997, *Mol. Endocrinol.* 11: 415-423 [changes in calcium concentration measured using the aequorin assay]; Offermanns & Simon, 1995, *J. Biol. Chem.* 270: 15175-15180 [changes in inositol phosphate levels]). Depending upon the cells in which heteromers of GABA_BR1a and GABA_BR2 are expressed, and thus the G-proteins with which the

functional GABA_B receptor thus formed is coupled, certain of such methods may be appropriate for measuring the functional responses of such functional GABA_B receptors. It is well within the competence of one skilled in the art to select the appropriate method of measuring functional responses for a given experimental system.

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The term "compound", "test compound", "agent" or "candidate agent" as used herein can be any type of molecule, including for example, a peptide, a polynucleotide, or a small molecule that one wishes to examine for their activity as GABA_B receptor agonist, and wherein said agent may provide a therapeutic advantage to the subject receiving it. The candidate agents can be administered to an individual by various routes, including, for example, orally or parenterally, such as intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally, intrarectally, intracisternally or by passive or facilitated absorption through the skin, using for example a skin patch or transdermal iontophoresis, respectively. Furthermore the compound can be administered by injection, intubation or topically, the latter of which can be passive, for example, by direct application of an ointment, or active, for example, using a nasal spray or inhalant, in which case one component of the composition is an appropriate propellant. The route of administration of the compound will depend, in part, on the chemical structure of the compound. Peptides and polynucleotides, for example, are not particularly useful when administered orally because they can be degraded in the digestive tract. However, methods for chemically modifying peptides, for example rendering them less susceptible to degradation are well known and include for example, the use of D-amino acids, the use of domains based on peptidomimetics, or the use of a peptoid such as a vinylogous peptoid.

25 The agent used in the screening method may be used in a pharmaceutically acceptable carrier. See, e.g., *Remington's Pharmaceutical Sciences*, latest edition, by E.W. Martin Mack Pub. Co., Easton, PA, which discloses typical carriers and conventional methods of preparing pharmaceutical compositions that may be used in conjunction with the preparation of formulations of the agents and which is
30 incorporated by reference herein.

Cells

As already outlined above, the present invention provides a cell line stably transfected with expression vectors that direct the expression of the GABA_B receptor subunits GABA_BR1a and GABA_BR2 as defined hereinbefore. In particular CHO cells transfected with said expression vectors. Such expression vectors are routinely

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constructed in the art of molecular biology and may involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, which may be necessary, and which are positioned in the correct orientation, in order to allow for protein expression. Generally, any system or vector suitable to maintain, propagate or
5 express polynucleotides to produce a polypeptide in a host may be used. The appropriate nucleotide sequence, i.e. the polynucleotide sequences encoding either the human GABA_BR1a or GABA_BR2 subunit as defined hereinbefore, may be inserted into an expression system by any of a variety of well-known and routine techniques such as for example those set forth in Current Protocols in Molecular Biology, Ausbel et al.
10 eds., John Wiley & Sons, 1997.

In a particular embodiment the CHO cells according to the invention are cotransfected with the commercially available expression vectors pcDNA3.1 comprising the polynucleotide sequences encoding for human GABA_BR1a (SEQ ID
15 No.:1) and human GABA_BR2 (SEQ ID No.: 3) respectively. More preferably the present invention provides a hGABA_BR1a/GABA_BR2 CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with the accession number LMBP 6046CB. This cell line is characterized in that the functional GABA_B receptor in this CHO cell line
20 has both a low and a high affinity binding site for GABA_B receptor agonist. Using the cell line according to the invention, will not only allow compound screening, but also provides a useful tool for the characterization of the nature of the compound-receptor interaction, i.e. does it interact with the low or high affinity agonist binding site of the GABA_B receptor.

25 For further details in relation to the preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, see for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press.

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Assays

The present invention also provides an assay for a compound capable of interacting with the functional GABA_B receptor of the present invention, which assay comprises:
35 providing the GABA_B receptor expressed by the hGABA_BR1a/GABA_BR2 CHO cell line of the present invention, contacting said receptor with a putative binding

compound; and determining whether said compound is able to interact with said receptor.

5 In one embodiment of the assay, the receptor or subunits of the receptor may be employed in a binding assay. Binding assays may be competitive or non-competitive. Such an assay can accommodate the rapid screening of a large number of compounds to determine which compounds, if any, are capable of binding to the polypeptides.

10 Within this context, the present invention provides a method to identify whether a test compound binds to an isolated GABA_B receptor protein of the present invention, and is thus a potential agonist or antagonist of the GABA_B receptor, said method comprising;

- a) contacting cells expressing a functional GABA_B receptor, wherein such cells do not normally express the GABA_B receptor, with the test compound in the presence and absence of a compound known to bind the GABA_B receptor, and
- 15 b) determine the binding of the test compound to the GABA_B receptor using the compound known to bind to the GABA_B receptor as a reference.

Binding of the test compound or of the compound known to bind to the GABA_B receptor, hereinafter also referred to as reference compound, is assessed using art-known methods for the study of protein-ligand interactions. For example, such binding
20 can be measured by employing a labeled substance or reference compound. The test compound or reference compound can be labeled in any convenient manner known in the art, *e.g.* radioactively, fluorescently or enzymatically. In a particular embodiment of the aforementioned method, the compound known to bind to the GABA_B receptor, a.k.a. the reference compound is detectably labeled, and said label is used to determine
25 the binding of the test compound to the GABA_B receptor. Said reference compound being labeled using a radiolabel, a fluorescent label or an enzymatic label, more preferably a radiolabel. In a more particular embodiment, the present invention provides a method to identify whether a test compound binds to an isolated GABA_B receptor protein, said method comprising the use of a compound known to bind to the
30 GABA_B receptor, wherein said reference compound is selected from the group consisting of ³H-GABA, ³H-baclofen, ³H-3-APPA, ³H-CGP542626 and ³H-SCH50911.

Subsequently, more detailed assays can be carried out with those compounds found to bind, to further determine whether such compounds act as agonists or antagonists of the
35 polypeptides of the invention.

Thus, in a further embodiment the present invention provides a method to identify GABA_B receptor agonists said method comprising,

- a) exposing cells expressing a functional GABA_B receptor, wherein such cells do not normally express the GABA_B receptor, to a labeled agonists of GABA_B in the presence and absence of the test compound, and
- b) determine the binding of the labeled agonist to said cells, where if the amount of binding of the labeled agonist is less in the presence of the test compound, then the compound is a potential agonist of the GABA_B receptor. As already specified for the general binding assay above, the binding of the GABA_B receptor agonists is assessed using art-known methods for the study of protein-ligand interactions. The label is generally selected from a radioactive label, a fluorescent label or an enzymatic label, in particular a radiolabel wherein the agonist is selected from the group consisting of ³H-GABA, ³H-baclofen and ³H-3-APPA.

- Similarly, the present invention provides a method to identify GABA_B receptor antagonists said method comprising,
- a) exposing cells expressing a functional GABA_B receptor, wherein said cells do not normally express the GABA_B receptor, to a labeled antagonist of GABA_B in the presence and absence of the test compound, and
- b) determine the binding of the labeled antagonist to said cells, where if the amount of binding of the labeled antagonist is less in the presence of the test compound, then the compound is a potential antagonist of the GABA_B receptor. As already specified for the general binding assay above, the binding of the GABA_B receptor antagonists is assessed using art-known methods for the study of protein-ligand interactions. The label is generally selected from a radioactive label, a fluorescent label or an enzymatic label, in particular a radiolabel wherein the antagonist is selected from the group consisting of ³H-CGP542626 and ³H-SCH50911.

- In an alternative embodiment of the present invention, the aforementioned binding assays are performed on a cellular composition, i.e a cellular extract, a cell fraction or cell organelles comprising a GABA_B receptor as defined hereinbefore. More in particular, the aforementioned binding assays are performed on a cellular composition, i.e. a cellular extract, a cell fraction or cell organelles comprising a GABA_B receptor as defined hereinbefore, wherein said cellular composition, i.e. cellular extract, cell fraction or cell organelles, is obtained from cells expressing a functional GABA_B receptor, wherein said cells do not normally express the GABA_B receptor. More preferably, the cellular composition, i.e. cellular extract, cell fraction or cell organelles, is

obtained from the hGABA_BR1a/GABA_BR2 CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with the accession number LMBP 6046CB.

- It is accordingly, an object of the present invention to provide a method for
- 5 identifying a compound as a GABA_B receptor agonist or antagonist, said method comprising;
- a) administering the compound to a cellular composition of cells expressing a functional GABA_B receptor, wherein said cells do not normally express the GABA_B receptor, in the presence of a detectably labeled agonist or antagonist of the GABA_B
- 10 receptor; and
- b) determine the binding of the labeled agonist or antagonist to said cellular composition,
- where if the amount of binding of the labeled agonist or antagonist is less in the presence of the test compound, then the compound is a potential agonist respectively
- 15 antagonist of the GABA_B receptor.
- As already specified for the general binding assay above, the binding of the GABA_B receptor agonist or antagonist is assessed using art-known methods for the study of protein-ligand interactions. The label is generally selected from a radioactive label, a fluorescent label or an enzymatic label, in particular a radiolabel wherein the agonist is
- 20 selected from the group consisting of ³H-GABA, ³H-baclofen and ³H-3-APPA and the antagonist is selected from the group consisting of ³H-CGP542626 and ³H-SCH50911.
- In a more specific embodiment the aforementioned binding assays are performed on a cellular composition consisting of the membrane fraction of cells according to the invention, in particular on membrane fractions of the hGABA_BR1a/GABA_BR2 CHO
- 25 cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with the accession number LMBP 6046CB, using one or more of the aforementioned radiolabeled agonist and/or antagonists.
- 30 In a further embodiment the present invention provides a functional assay for identifying compounds that modulate the GABA_B-receptor activity in the cells according to the invention. Such an assay is conducted using the cells of the present invention, i.e. cotransfected with the human GABA_BR1a and human GABA_BR2 subunits. The cells are contacted with at least one reference compound wherein the ability of said
- 35 compound to modulate the GABA_B-receptor activity is known. Thereafter, the cells are contacted with a test compound and determined whether said test compound modulates the activity of the GABA_B receptor compared to the reference compound. A "reference

compound” as used herein refers to a compound that is known to bind and/or to modulate the GABA_B receptor activity.

5 A compound or a signal that “modulates the activity” of a polypeptide of the invention refers to a compound or a signal that alters the activity of the polypeptide so that it behaves differently in the presence of the compound or signal than in the absence of the compound or signal. Compounds affecting modulation include agonists and antagonists. An agonist of the GABA_B receptor encompasses a compound such as GABA, baclofen and 3 - APPA which activates GABA_B receptor function.
10 Alternatively, an antagonist includes a compound that interferes with GABA_B receptor function. Typically, the effect of an antagonist is observed as a blocking of agonist-induced receptor activation. Antagonists include competitive as well as non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts with or near the site specific for agonist binding. A non-competitive antagonist or
15 blocker inactivates the function of the receptor by interacting with a site other than the agonist interaction site.

In one embodiment the present invention provides a method for identifying compounds that have the capability to modulate GABA_B receptor activity, said method comprising;
20 a) contacting cells expressing a functional GABA_B receptor, wherein said cells do not normally express a functional GABA_B receptor, with at least one reference compound, under conditions permitting the activation of the GABA_B receptor;
b) contacting the cells of step a) with a test compound, under conditions permitting the activation of the GABA_B receptor, and
25 c) determine whether said test compound modulates the GABA_B receptor activity compared to the reference compound.

Methods to determine the capability of a compound to modulate the GABA_B receptor activity are based on the variety of assays available to determine the functional response of G-protein coupled receptors (see above) and in particular on assays to determine the
30 changes in potassium currents, changes in calcium concentration, changes in cAMP and changes in GTPγS binding. Conditions permitting the activation of the GABA_B receptor generally known in the art, for example in case of antagonist screening these conditions comprise the presence of a GABA_B receptor agonist in the assay system.
35 Typical GABA_B receptor agonists used in these activity assays are GABA, baclofen or 3-APPA. More particular in the GTPγS assay as outlined herein below, GABA is used

to activate the GABA_B receptor in order to assess the capability of a test compound to inactivate the GABA_B receptor protein.

5 In the aforementioned assay an increase of GTPγS binding in the presence of the test compound is an indication that the compound activates the GABA_B receptor activity, and accordingly that said test compound is a potential agonist of the GABA_B receptor protein. A decrease of GTPγS binding in the presence of the test compound is an indication that the compound inactivates the GABA_B receptor protein and accordingly that said test compound is a potential antagonist of the GABA_B receptor protein.

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Particularly preferred types of assays include binding assays and functional assays which may be performed as follows:

Binding assays

15 Over-expression of the GABA_B receptor expressed by the hGABA_BR1a/GABA_BR2 CHO cell line of the present invention may be used to produce membrane preparations bearing said receptor (referred to in this section as GABA_B binding receptor for convenience) for ligand binding studies. These membrane preparations can be used in conventional filter-binding assays (eg. Using Brandel filter assay equipment) or in high
20 throughput Scintillation Proximity type binding assays (SPA and Cytostar-T flashplate technology; Amersham Pharmacia Biotech) to detect binding of radio-labelled GABA_B ligands (including ³H-GABA, ³H-baclofen, ³H-3-APPA, ³H-CGP542626, ³H-SCH50911) and displacement of such radio-ligands by competitors for the binding site. Radioactivity can be measured with Packard Topcount, or similar instrumentation,
25 capable of making rapid measurements from 96-, 384-, 1536- microtitre well formats. SPA/Cytostar-T technology is particularly amenable to high throughput screening and therefore this technology is suitable to use as a screen for compounds able to displace standard ligands.

30 Another approach to study binding of ligands to GABA_B binding receptor protein in an environment approximating the native situation makes use of a surface plasmon resonance effect exploited by the Biacore instrument (Biacore). GABA_B binding receptor in membrane preparations or whole cells could be attached to the biosensor chip of a Biacore and binding of ligands examined in the presence and
35 absence of compounds to identify competitors of the binding site.

Functional assays

Since GABA_B receptors belong to the family G-protein coupled receptors that are coupled to GIRK (inward rectifying potassium channels), potassium ion flux should result on activation of these receptors. This flux of ions may be measured in real time using a variety of techniques to determine the agonistic or antagonistic effects of particular compounds. Therefore, recombinant GABA_B binding receptor proteins expressed in the cell lines of the present invention can be characterised using whole cell and single channel electrophysiology to determine the mechanism of action of compounds of interest. Electrophysiological screening, for compounds active at GABA_B binding receptor proteins, may be performed using conventional electrophysiological techniques and when they become available, novel high throughput methods currently under development.

Given the presynaptic effect of GABA_B receptor activation on Ca²⁺ channels, in an alternative functional screen the modulatory effect of a compound is assessed through the changes in intracellular calcium. Calcium fluxes are measurable using several ion-sensitive fluorescent dyes, including fluo-3, fluo-4, fluo-5N, fura red and other similar probes from suppliers including Molecular Probes. The inhibition of calcium influx as a result of GABA_B receptor activation can thus be characterised in real time, using fluorometric and fluorescence imaging techniques, including fluorescence microscopy with or without laser confocal methods combined with image analysis algorithms.

Another approach is a high throughput screening assay for compounds active as either agonists or modulators which affect calcium transients. This assay is based around an instrument called a **FLuorescence Imaging Plate Reader ((FLIPR®)**, Molecular Devices Corporation). In its most common configuration, it excites and measures fluorescence emitted by fluorescein-based dyes. It uses an argon-ion laser to produce high power excitation at 488 nm of a fluorophore, a system of optics to rapidly scan the over the bottom of a 96-/384-well plate and a sensitive, cooled CCD camera to capture the emitted fluorescence. It also contains a 96-/384-well pipetting head allowing the instrument to deliver solutions of test agents into the wells of a 96-/384-well plate. The FLIPR assay is designed to measure fluorescence signals from populations of cells before, during and after addition of compounds, in real time, from all 96-/384-wells simultaneously. The FLIPR assay may be used to screen for and characterise compounds functionally active at the hGABA_BR1a/GABA_BR2 CHO cell line.

A high throughput screening assay, specifically usefull to identify GABA_B agonists could consist of an arrangement wherein hGABA_BR1a/GABA_BR2 CHO cells, are loaded with an appropriate fluorescent dye, incubated with a test compound and
5 after sufficient time to allow interaction (8 – 24 hours, typically 12-24 hours, in particular 24 hours.) the change in relative fluorescence units measured using an automated fluorescence plate reader such as FLIPR or Ascent Fluoroskan (commercially available from Thermo Labsystems, Brussel, Belgium).

10 In a further embodiment the functional assay is based on the change in GTPγS binding to the GABA_B binding receptor. In particular using a competition bindig assay to determine the displacement of radiolabelled GTPγS. In general, this method to identify GABA_B-receptor agonists comprises preparing a membrane fraction from cells
15 expressing the hGABA_BR1a/GABA_BR2 heterodimer af the present invention, contacting said membrane preparations with the compound to be tested in the presence of radiolabelled GTPγS, under conditions permitting the activation of the GABA_B receptor, and detecting GTPγS binding to the membrane fraction. An increase in GTPγS binding in the presence of the compound is an indication that the compound
20 activates the hGABA_BR1a/GABA_BR2 receptor. A decrease in GTPγS binding in the presence of the compound is an indication that the compound inactivates the hGABA_BR1a/GABA_BR2 receptor. Preferably this GTPγS binding assay is performed on membrane fractions obtained from the hGABA_BR1a/GABA_BR2 CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-
25 6046CB. Further, the conditions permitting the activation of the GABA_B receptor comprise the presence of a GABA_B receptor agonist, such as for example GABA, baclofen and 3-APPA in the assay system. In particular GABA.

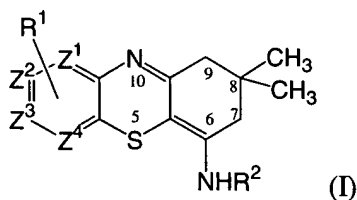
This and other functional screening assays will be provided in the examples hereinafter.

30

GABA_B receptor agonists

In a further aspect the present invention provides GABA_B receptor agonists identified using one of the aforementioned screening assays wherein said GABA_B receptor agonists are represented by the compounds of formula (I)

35



the *N*-oxide forms, the pharmaceutically acceptable addition salts and the stereochemically isomeric forms thereof, wherein

- $=Z^1-Z^2=Z^3-Z^4=$ represents a divalent radical selected from the group consisting of
- 5 $=N-CH=CH-N=$ (a), $=N-CH=N-CH=$ (b), $=CH-N=CH-N=$ (c)
 $=CH-CH=CH-CH=$ (d), $=N-CH=CH-CH=$ (e), $=CH-N=CH-CH=$ (f),
 $=CH-CH=N-CH=$ (g) and $=CH-CH=CH-N=$ (h);
- R^1 represents hydrogen, halo, hydroxyl, cyano, C_{1-6} alkyl, CF_3 , amino or mono- or di(C_{1-4} alkyl)amino;
- 10 R^2 represents hydrogen, C_{1-6} alkyl or hydroxycarbonyl- C_{1-6} alkyl-.

In particular those compounds of formula (I) wherein one or more of the following restrictions apply;

- (i) $=Z^1-Z^2=Z^3-Z^4=$ represents a divalent radical selected from the group
- 15 consisting of
 $=N-CH=CH-N=$ (a), $=N-CH=N-CH=$ (b), $=CH-N=CH-N=$ (c) and
 $=CH-CH=CH-CH=$ (d);
- (ii) R^1 represents halo, amino or mono- or di(C_{1-4} alkyl)amino;
- (iii) R^2 represents butyric acid

20

Also of interest are those compounds of formula (I) wherein;

- (i) R^1 is attached at position Z^1 ; and/or
- (ii) $=Z^1-Z^2=Z^3-Z^4=$ represents (a), (b) or (d), more preferably
 $=Z^1-Z^2=Z^3-Z^4=$ represents (d).

25

As used in the foregoing definitions and hereinafter, halo is generic to fluoro, chloro, bromo and iodo; C_{1-4} alkyl defines straight and branched chain saturated hydrocarbon radicals having from 1 to 4 carbon atoms such as, for example, methyl, ethyl, propyl, butyl, 1-methylethyl, 2-methylpropyl, 2,2-dimethylethyl and the like; C_{1-6} alkyl defines

30 straight and branched chain saturated hydrocarbon radicals having from 1 to 6 carbon atoms such as, for example, pentyl, hexyl, 3-methylbutyl, 2-methylpentyl and the like.

The pharmaceutically acceptable addition salts as mentioned hereinabove are meant to comprise the therapeutically active non-toxic acid addition salt forms, which the compounds of formula (I), are able to form. The latter can conveniently be obtained by treating the base form with such appropriate acid. Appropriate acids comprise, for example, inorganic acids such as hydrohalic acids, e.g. hydrochloric or hydrobromic acid; sulfuric; nitric; phosphoric and the like acids; or organic acids such as, for example, acetic, propanoic, hydroxyacetic, lactic, pyruvic, oxalic, malonic, succinic (i.e. butanedioic acid), maleic, fumaric, malic, tartaric, citric, methanesulfonic, ethanesulfonic, benzenesulfonic, *p*-toluenesulfonic, cyclamic, salicylic, *p*-aminosalicylic, pamoic and the like acids.

The pharmaceutically acceptable addition salts as mentioned hereinabove are meant to comprise the therapeutically active non-toxic base addition salt forms which the compounds of formula (I), are able to form. Examples of such base addition salt forms are, for example, the sodium, potassium, calcium salts, and also the salts with pharmaceutically acceptable amines such as, for example, ammonia, alkylamines, benzathine, *N*-methyl-D-glucamine, hydrabamine, amino acids, e.g. arginine, lysine.

Conversely said salt forms can be converted by treatment with an appropriate base or acid into the free acid or base form.

The term addition salt as used hereinabove also comprises the solvates which the compounds of formula (I), as well as the salts thereof, are able to form. Such solvates are for example hydrates, alcoholates and the like.

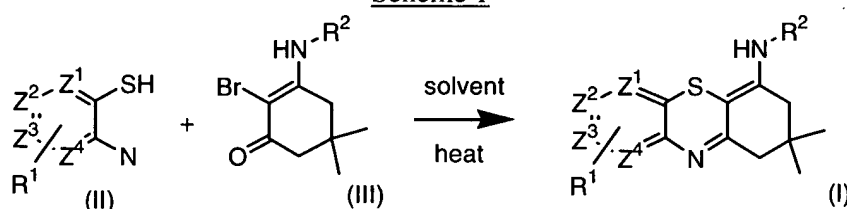
The term stereochemically isomeric forms as used hereinbefore defines the possible different isomeric as well as conformational forms which the compounds of formula (I), may possess. Unless otherwise mentioned or indicated, the chemical designation of compounds denotes the mixture of all possible stereochemically and conformationally isomeric forms, said mixtures containing all diastereomers, enantiomers and/or conformers of the basic molecular structure. All stereochemically isomeric forms of the compounds of formula (I), both in pure form or in admixture with each other are intended to be embraced within the scope of the present invention.

The *N*-oxide forms of the compounds of formula (I), are meant to comprise those compounds of formula (I) wherein one or several nitrogen atoms are oxidized to the so-called *N*-oxide.

The 7,8-dihydro-phenothiazine derivatives of the present invention are generally prepared as described by Nemeryuk M.P. et al., *Khimiko-Farmatsevticheskii Zhurnal* (1985), 19(8), 964-968. In brief, the known ortho-amino substituted (hetero)arene-thiols (II), are condensed with an appropriate 2-bromo-5,5-dimethyl-3-oxo-cyclohex-1-enylamino derivative (III), by heating the two reactants in a suitable solvent, such as ethanol or N-methylpyrrolidone. Standard work-up and purification gives the desired products of formula I (Scheme 1).

10

Scheme 1

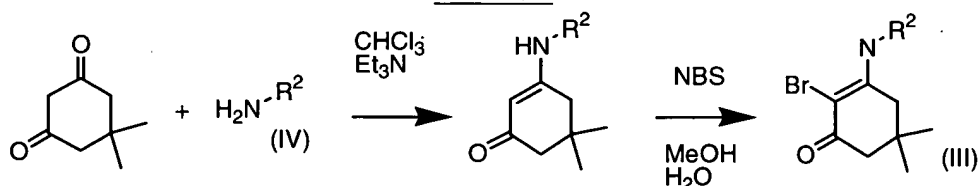


Wherein $=Z^1-Z^2=Z^3-Z^4=$, R¹ and R² are defined as for the compounds of formula (I) hereinbefore.

15 The appropriate 2-bromo-5,5-dimethyl-3-oxo-cyclohex-1-enylamino derivatives (III) can generally be obtained by amination of 5,5-dimethyl-1,3-cyclohexanedione with the appropriate amine of general formula (IV) under art known amination conditions, followed by bromination with *N*-bromosuccinimide (Scheme 2).

20

Scheme 2

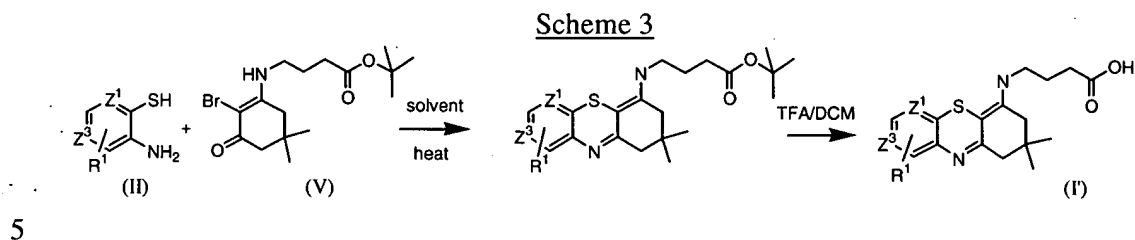


Wherein R² is defined as for the compounds of formula (I) hereinbefore.

25 For those compounds of formula (I) where R² represents butyric acid, hereinafter referred to as the compounds of formula (I'), the compounds are obtained by condensing the ortho-amino substituted (hetero)arene-thiol (II) with 4-(2-bromo-5,5-dimethyl-3-oxo-cyclohex-1-enylamino)-butyric acid or an ester derivative such as a *t*-butylester (V) using art known conditions, such as for example by heating the two reactants in a suitable solvent, such as ethanol or N-methylpyrrolidone. Standard work-up and purification gives the desired products, or the ester derivative, which can be

30

hydrolyzed under acidic or basic conditions to give the required butyric acids (I') (Scheme 3).



Further examples for the synthesis of compounds of formula (I) using the above mentioned synthesis method is provided in the experimental part hereinafter.

- 10 Where necessary or desired, any one or more of the following further steps in any order may be performed:
 - (i) removing any remaining protecting group(s);
 - (ii) converting a compound of formula (I) or a protected form thereof into a further compound of formula (I) or a protected form thereof;
 - 15 (iii) converting a compound of formula (I) or a protected form thereof into a *N*-oxide, a salt, a quaternary amine or a solvate of a compound of formula (I) or a protected form thereof;
 - (iv) converting a *N*-oxide, a salt, a quaternary amine or a solvate of a compound of formula (I) or a protected form thereof into a compound of formula (I) or a protected form thereof;
 - 20 (v) converting a *N*-oxide, a salt, a quaternary amine or a solvate of a compound of formula (I) or a protected form thereof into another *N*-oxide, a pharmaceutically acceptable addition salt a quaternary amine or a solvate of a compound of formula (I) or a protected form thereof.

25 It will be appreciated by those skilled in the art that in the processes described above the functional groups of intermediate compounds may need to be blocked by protecting groups.

- 30 Functional groups which it is desirable to protect include hydroxy, amino and carboxylic acid. Suitable protecting groups for hydroxy include trialkylsilyl groups (e.g. tert-butyldimethylsilyl, tert-butyldiphenylsilyl or trimethylsilyl), benzyl and tetrahydropyranyl. Suitable protecting groups for amino include tert-butyloxycarbonyl or

benzyloxycarbonyl. Suitable protecting groups for carboxylic acid include C₍₁₋₆₎alkyl or benzyl esters.

5 The protection and deprotection of functional groups may take place before or after a reaction step.

The use of protecting groups is fully described in 'Protective Groups in Organic Synthesis' 3rd edition, T W Greene & P G M Wutz, John Wiley & Sons Inc. (June 1999).

10

Additionally, the N-atoms in compounds of formula (I) can be methylated by art-known methods using CH₃-I in a suitable solvent such as, for example 2-propanone, tetrahydrofuran or dimethylformamide.

15 Some of the intermediates and starting materials as used in the reaction procedures mentioned hereinabove are known compounds and may be commercially available or may be prepared according to art-known procedures.

Method of Treatment

20 The present invention also provides the use of a compound identified as a GABA_B receptor activity modulator, using one of the aforementioned assays, in particular the compounds of formula (I) as described hereinbefore, in the manufacture of a medicament for the treatment an indication such as stiff man syndrome, gastroesophageal reflux, neuropathic pain, incontinence and treatment of cough and
25 cocaine addiction. In particular for use in the manufacture of a medicament to reduce transient lower esophageal sphincter relaxations (TLESR). It is thus an object of the present invention to provide a method for the treatment of a warm-blooded animal, for example, a mammal including humans, suffering from an indication such as stiff man syndrome, gastroesophageal reflux, neuropathic pain, incontinence and treatment of
30 cough and cocaine addiction, in particular TLESR.

Said method comprising administering to a warm-blooded animal in need thereof an effective amount of a compound identified as a GABA_B receptor modulator using a method according to the invention. In particular the systemic or topical administration of an effective amount of a compound according to the invention, to
35 warm-blooded animals, including humans.

Such agents may be formulated into compositions comprising an agent together with a pharmaceutically acceptable carrier or diluent. The agent may in the form of a physiologically functional derivative, such as an ester or a salt, such as an acid addition salt or basic metal salt, or an N or S oxide. Compositions may be formulated for any
5 suitable route and means of administration. Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal, inhalable, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. The choice of carrier or diluent will of course depend on the proposed
10 route of administration, which, may depend on the agent and its therapeutic purpose. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared
15 by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

For solid compositions, conventional non-toxic solid carriers include, for example,
20 pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium carbonate, and the like may be used. The active compound as defined above may be formulated as suppositories using, for example, polyalkylene glycols, acetylated triglycerides and the like, as the carrier. Liquid pharmaceutically administrable compositions can, for
25 example, be prepared by dissolving, dispersing, etc, an active compound as defined above and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying
30 agents, pH buffering agents and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Gennaro et al., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 18th
35 Edition, 1990.

The composition or formulation to be administered will, in any event, contain a quantity of the active compound(s) in an amount effective to alleviate the symptoms of the subject being treated.

- 5 Dosage forms or compositions containing active ingredient in the range of 0.25 to 95% with the balance made up from non-toxic carrier may be prepared.

For oral administration, a pharmaceutically acceptable non-toxic composition is formed by the incorporation of any of the normally employed excipients, such as, for example,
10 pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, sodium crosscarmellose, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium carbonate, and the like. Such compositions take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained release formulations and the like. Such compositions may contain 1%-95% active ingredient, more
15 preferably 2-50%, most preferably 5-8%.

Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension
20 in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate,
25 triethanolamine sodium acetate, etc.

The percentage of active compound contained in such parental compositions is highly dependent on the specific nature thereof, as well as the activity of the compound and the needs of the subject. However, percentages of active ingredient of 0.1% to 10% in
30 solution are employable, and will be higher if the composition is a solid which will be subsequently diluted to the above percentages. Preferably, the composition will comprise 0.2-2% of the active agent in solution.

35 Throughout this description the terms "standard methods", "standard protocols" and "standard procedures", when used in the context of molecular biology techniques, are to be understood as protocols and procedures found in an ordinary laboratory

manual such as: Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994, or Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

5

This invention will be better understood by reference to the Experimental Details that follow, but those skilled in the art will readily appreciate that these are only illustrative of the invention as described more fully in the claims that follow thereafter. Additionally, throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

10

EXPERIMENTAL PART

I SYNTHESIS OF GABA_B AGONISTS

15

In the procedures described hereinafter the following abbreviations were used : "DIPE" stands for diisopropylether; "EtOAc" stands for ethyl acetate.

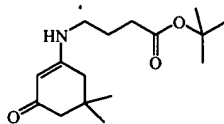
For some chemicals the chemical formula was used, e.g. CH₃CN for acetonitrile, NH₃ for ammonia, CH₂Cl₂ for dichloromethane, MgSO₄ for magnesium sulfate, and HCl for hydrochloric acid.

20

A. Preparation of the intermediates

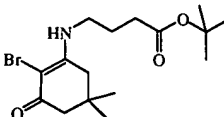
Example A.1

Preparation of



intermediate 1

and



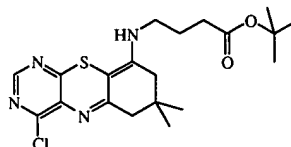
intermediate 2

4-Aminobutanoic acid 1,1-dimethylethyl ester [50479-22-6] (14g, 0.087mol) and 5,5-dimethyl-1,3-cyclohexanedione [126-81-8] (12.26g, 0.087mol) were dissolved in trichloromethane (250 ml) and *N,N*-diethylethanamine (0,5ml) was added. The reaction mixture was stirred for 3 days and subsequently washed with three portions of 250ml of water. The organic layer was dried on MgSO₄ and concentrated under reduced pressure. The residue was recrystallised in DIPE/ CH₃CN to give 18.6g (76%) of intermediate 1.

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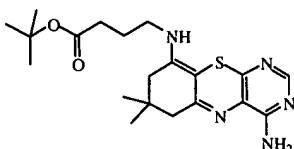
This product was taken up in methanol (250 ml) and water (100 ml). 1-Bromo-2,5-pyrrolidinedione (11.8g, 0.066 mol) was added portionwise over a 30 minutes period. After stirring for an additional hour, 500 ml water was added. The mixture was extracted with three portions of dichloromethane. The combined organic layers were dried on MgSO_4 and concentrated under reduced pressure to yield 22g (92%) of intermediate 2.

In a similar way was also prepared :



intermediate 3

Example A.2

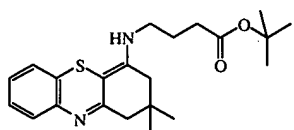


Preparation of

intermediate 4

A mixture of 5,6-diamino-4(1H)-pyrimidinethione [2846-89-1] (0.0027 mol) and intermediate 2 (0.0027 mol) in ethanol (q.s.) was stirred for 2 hours at 85°C. The reaction mixture was filtered and the solvent was evaporated. The residue was purified by high-performance liquid chromatography. The product fractions were collected and the solvent (CH_3CN) was evaporated. The aqueous layer was extracted with EtOAc. The organic layer was separated, dried (MgSO_4), filtered and the solvent was evaporated, yielding 0.400 g (30%) of intermediate 4.

Example A.3



Preparation of

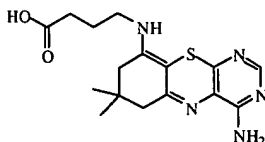
intermediate 5

A mixture of 2-aminobenzenethiol [137-07-5] (0.004 mol) and intermediate 2 (0.004 mol) in 1-methyl-2-pyrrolidinone [872-50-4] (15 ml) was stirred for 1 hour at 140°C. The reaction mixture was cooled and the layers were separated with EtOAc/ $\text{H}_2\text{O}(\text{NH}_3)$. The organic layer was dried (MgSO_4), filtered and the solvent was evaporated. The residue was purified by high-performance liquid chromatography. The product fractions were collected and the solvent (CH_3CN) was evaporated. The aqueous layer was extracted with EtOAc and then the organic layer was dried (MgSO_4), filtered off and the solvent was evaporated, yielding 0.6 g (40 %) of intermediate 5.

B. Preparation of the compounds

Example B.1

Preparation of

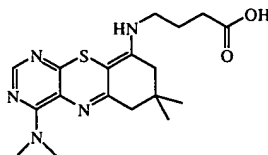


compound 2

A mixture of intermediate 4 (0.001 mol) in trifluoroacetic acid (5 ml) and dichloromethane (5 ml) was stirred for 1 hour at room temperature. The reaction mixture was dried under a stream of nitrogen. The resulting residue was suspended in diethyl ether. The desired product was filtered off and dried (vacuo) at 30°C, yielding 0.120 g (23 %) of trifluoroacetic acid salt of compound 2.

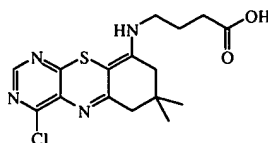
In a similar way were also prepared :

The hydrobromic acid salt of



compound 1

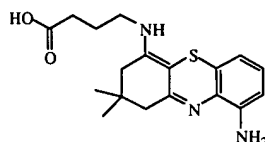
and the trifluoroacetic acid salt of



compound 4

10 Example B.2

Preparation of



compound 3

A mixture of intermediate 5 (0.00155 mol) in trifluoroacetic acid (5 ml) and dichloromethane (5 ml) was stirred for 20 hours at room temperature. The reaction mixture was dried under a stream of nitrogen. The resulting residue was solidified in diethyl ether. The desired product was filtered off and dried (vacuo) at 30°C, yielding 0.320 g (67 %) of trifluoroacetic acid salt of compound 3.

II DEVELOPMENT OF GABA_B-CHO-K1 CELLS

MATERIAL AND METHODS

20

Permanent transfection of GABABR1a and GABABR2 in CHO-K1 cells using Lipofectamine PLUS:

CHO-cells were transfected with hGABABR1a/pcDNA3.1. Monoclonal stable R1a-expressing cells were transfected with hGABABR2/pcDNA3.1Hygro+. Selection of clones occurred with 800 µg geneticin + 800 µg hygromycin/ml.

5 *Membrane preparation:*

Butyrate-stimulated (5 mM final) cells were scraped, after a short rinse with PBS, in 50 mM TrisHCl pH7.4 and centrifuged at 23500 g for 10 min. at 4°C. The pellet was homogenised in 5 mM TrisHCl pH 7.4 by Ultra-Turrax (24000 rpm) followed by centrifugation at 30000 g for 20 min. at 4°C. The resulting pellet was resuspended in 10 50 mM TrisHCl pH 7.4 and rehomogenised. Protein concentration was determined using the Bradford method.

GTPγ³⁵S activation assay:

10 µg membrane prep was incubated in 250 µl in 20 mM Hepes pH 7.4, 100 mM NaCl, 3 mM MgCl₂, 0.25 nM GTPγ³⁵S, 3 µM GDP, 10 µg saponin/ml, with or without 1mM GABA (basal activity in absence of baclofen) at 37°C for 20 min. Filtration was carried out onto 96-well GF/B filter plate in Harvester (Packard). Filters were rinsed 6 times with cold 10 mM phosphate buffer pH 7.4, and dried overnight before addition of 30 µl Microscint O, and measurement in Topcount (Packard, 20 1min./well).

3H-agonist binding:

30 - 60 µg membrane prep was incubated in 50 mM TrisHCl pH 7.4, 2.5 mM CaCl₂, 10 nM 3H-GABA or 20 nM 3H-baclofen in 500 µl at 20°C. Non-specific binding was 25 determined in the presence of 100 µM baclofen. After 90 minutes the mixture was transferred onto 96-well GF/B filterplate by Harvester (Packard). Filters were rinsed 6 times with cold 50 mM TrisHCl pH 7.4, 2.5 mM CaCl₂, and dried overnight before addition of 30 µl Microscint O, and measurement in Topcount (Packard, 1min./well).

30 RESULTS

GTPγ³⁵S activation assay

In membranes of stably hGABABR1a-transfected CHO-cells, we measured binding of the antagonist 3H-CGP54626. hGABABR2 was co-transfected in those R1a-clones 35 with the highest antagonist binding. After subcloning stable clones were obtained

showing functional activity in GTP γ 35S-binding assay upon stimulation of membranes by GABA, wherein said activity was potentiated in the presence of the positive modulator CGP7930 (Urwyler S., *et al.*, 2001, Molecular Pharmacology60:963-971) (fig. 1).

5

Agonist Filter Binding Assay

An agonist filter binding assay has been developed in 96-well GF/B filterplate. The IC50 of known agonists and antagonists was determined (fig.2). While the stable hGABA_BR1a or the transient hGABA_BR2 monomeric GABA_B receptor expressing
 10 cells did not show any binding to the agonists 3H-GABA or 3H-baclofen (data not shown), unexpectedly, in our hGABABR1a/R2 heterodimeric clone agonist binding was detected with both ligands . The Kd for 3H-baclofen, 3H-GABA, and 3H-CGP54626 was determined in saturation experiments and compared well with published results obtained with tissue preparations (table 1).

15 Table 1

³H-baclofen

	Rat	132 nM	(Hill & Bowery, 1981)
	Dog cortex	28 nM	(J&JPRD, 2000)
20	hGABA _B R1aR2/CHO	30 nM	(our data, n=2))

³H-GABA

	Rat	77 nM	(Hill & Bowery, 1983)
	Rat	15-30 nM	(Cross & Horton, 1988)
25	Pig	26 nM	(Facklam & Bowery, 1993)
	Human	20-30 nM	(Cross & Horton, 1988)
	hGABA _B R1aR2/CHO	10-30 nM	(our data, n=6)

³H-CGP54626

30	Rat	1.5 nM	(Bittiger et al., 1993)
	Pig	1.35 nM	(Facklam & Bowery, 1993)
	hGABA _B R1aR2/CHO	1.5 nM	(Green et al., 1993)
	hGABA _B R1aR2/CHO	2.78 nM	(our data, n=1)

35 The order of potency for agonists was AMPA > GABA > baclofen, and for antagonists CGP54626 > SCH50911 (fig.2). The obtained IC50s were reproducible between different membrane preparations (fig.3)

Upon full library screening we identified some compounds with binding and signal transduction properties with comparable potencies as the reference compounds GABA and baclofen (table 2).

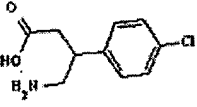
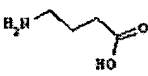
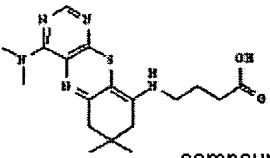
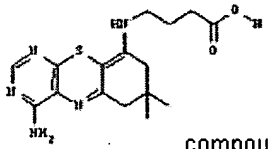
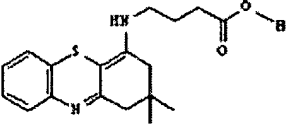
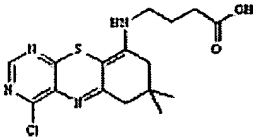
	BINDING ASSAY	SIGNAL TRANSDUCTION
	³ H-GABA binding	GTPγS binding
Chemistry	pIC50	%Effect at 10 μM
Reference compounds		
 baclofen	6.90775	78.7021
 GABA	8.06026	79.2906
HTS hits		
 compound 1	7.1875	45.7275
 compound 2	6.82	40.95
 compound 3	6.43	24.44
 compound 4	6.87	61.93

Table 2 : pIC₅₀ and % effect in the GABA ligand binding, and GTPγS signal transduction assays for reference compounds and HTS hits.

5 *Agonist centrifugation Binding Assay* In an alternative binding assay the non-bound ligand was separated from the membranes by centrifugation instead of filtration. The assay was performed according to the earlier described filter binding assay, with the difference that the non-bound ligand was separated from the membranes by centrifugation in a microcentrifuge at 12500 rpm for 10 minutes. The supernatant was discarded, the pellet was rinsed with washing buffer and dissolved in 200 µl water.
10 Scintillation fluid was added and the bound ³H-GABA measured in Topcount (Packhard, 1 min./well).

In a saturation assay using increasing concentrations of ³H-GABA (1 – 400 nM final) I was found that the GABA_B receptor expressed by the hGABA_BR1a/GABA_BR2 CHO cell line, possess a low and a high affinity agonist binding site. Results of the saturation
15 and scatchard analysis are summarized in Table 3. When the saturation assay was performed in the presence of 10 µM of the GABA_B antagonist CGP54626 or one of the GABA_B agonist of the present invention (compound 1), the ³H-GABA binding to both the high and the low affinity site was blocked (figure 4a, b).

20 Table 3

	Mean (n=5)	SD
	nM	nM
Bmax 1	0.19	0.05
Kd 1	9.4	3.1
Bmax 2	0.76	0.24
Kd 2	401	224

DISCUSSION

25 To our knowledge, no earlier reports were made in literature of recombinant hGABA_B receptor, showing agonist binding with a high and low affinity binding site in a filter binding assay. An HTS agonist filter binding screen has been developed using ³H-GABA. We found reproducible Ki values for known agonists and antagonists, independent of the membrane preparation.

It has in addition been demonstrated that the recombinant GABA_B receptor has two agonist binding sites. One high affinity and one low affinity binding site. It is to be expected that high affinity agonists of the GABA_B receptor will elicit a different response compared to the low affinity agonists. Hence, the cell line of the present
5 invention not only allows to identify GABA_B receptor agonists, but also provides a useful tool to characterize the nature of the compound receptor interaction.